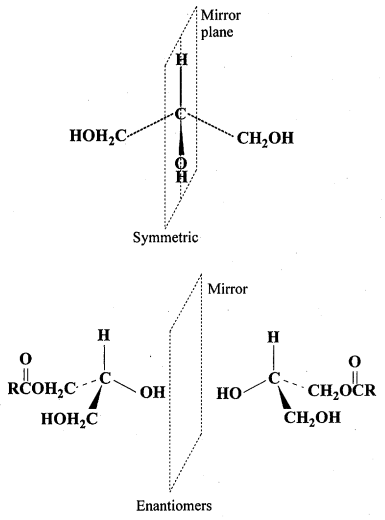
Lipase specificities:
Potential application in lipid bioconversions

Lipases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of acylglycerols. Because this reaction is reversible, it also can be used for the synthesis of such compounds (Figure 1). In addition, lipases are used widely in a variety of other reactions that can include esterification of acids with alcohols (1), oil and fat interesterifications (2) (Figure 1), as well as reactions leading to the synthesis of products such as sugar esters (3) or fatty amides (4,5). Moreover, because of their capability to preserve their catalytic activity in organic solvents (6), the use of lipases in industrial processes is becoming increasingly attractive (7).

Lipases are ubiquitous in nature, being present in the animal, microbial, and plant kingdoms. Animal lipases are found in several different organs. Examples include pancreatic, gastric, and pregastric lipases which are secreted by lingual and pharyngeal tissues. Plant lipases, found in several species (8), are less studied but are of interest because they often display a particular acylspecificity that is dependent upon the fatty acid composition and distribution of the triacylglycerols associated with a given species. The most extensively studied lipases are those from microbial sources probably because they are more readily available and exhibit several interesting properties. For example, they are more stable than plant or animal lipases and often show an ability to differentiate between enantiomers (see glossary of terms) of chiral molecules. This latter feature makes microbial lipases increasingly important for the kinetic resolution of chiral compounds that

Stereoisomer glossary



To understand stereoisomerisms, the first step is to explore the nature of symmetry as applied to molecular shapes. An object, or molecule, is symmetrical if, when a plane is passed through the center of the object, the reflection of one side of the object or molecule in that plane is identical to that of the other side. Such a plane of symmetry is often called a mirror plane. A tetrahedral carbon with at least two identical substituents has a plane of symmetry lying between the identical substituents. Inspection of the glycerol molecule shows that it possesses a plane of symmetry.

An object without a plane of symmetry is termed "asymmetric." A tetrahedral carbon with all four attached groups being different is asymmetric also; such carbon atoms are called asymmetric carbons. This condition is shown by inspection of the monoacylglycerol (MAG) structure. The two nonsuperimposable mirror image structures shown are referred to as mirror image isomers. Asymmetric compounds in nature are virtually always present as only one mirror image isomer.

From the foregoing, the following brief definitions can be provided for some of the stereochemical terms used in this article.

Stereoisomers: compounds that have the same sequence of covalent bonds, but which differ in the relative disposition of their atoms in space.

Oxidation–Protein Interaction *in vivo*, by H. Knapp, University of Iowa, United States.

• *Minor constituents, contaminants.* The following papers have been accepted thus far: Red Palm Oil—A New Vegetable Oil Containing Carotenoids and Other Natural Antioxidants, by Yuen-May Choo, Palm Oil Research Institute of Malaysia, Malaysia; Dolichols and Polyprenols in Palm Oil, by K.K. Carroll and N. Guthrie, University of Western Ontario, Canada; Oxidation of Oil During Frying, by W.W. Nawar, University of Massachusetts, United States; and Minor Components from Rice Bran Oil, by Akio Kato, Akio Tanaka, National Chemical Laboratory for Industry; and Masakazu Yamaok, National Institute of Bioscience and Human Technology, Japan.

• *Oils and fats in human nutrition.* The presentation on tocotrienols and cancer, by K.K. Carroll, University of Western Ontario, Canada, will be a keynote lecture. Additional technical presentations will include Dietary Fat and Its Influence on Energy Balance, by Philip James, The Rowett Research Institute, Scotland; Palm Oil and Human Nutrition, by Mohamad Amr Hussein, National Nutrition Institute, Egypt; Effect of Vitamin E and β-Carotene on Lymphoma Cells, by Muhammad Anwar Waqar, Aga Khan University, Pakistan; and Tocotrienols from Palm Oil Inhibit the Growth of Estrogen Responsive Human Breast Cancer Cells, by N. Kalanithi, Palm Oil Research Institute of Malaysia; R. Stephen, R. Dils, and P. Dabre, University of Reading, United Kingdom.

• *Oleochemicals.* The plenary lecture will be on the oleochemical outlook in the twenty-first century, presented by Yusof Basiron, Palm Oil Research Institute of Malaysia, Malaysia. Technical papers added include Alternate Processes for the Production of Methyl Ester Sulfonate, by Keith D. Hovda, Chemithon, United States; Softening Performance and Physicochemical Properties of Cationic–Anionic Mixed Surfactant System, by Hiroo Miyasaka, Jum Yokoyama, Shuichi Nihei, and Teruhsia Satsuki, Lion Corporation, Japan; Methyl Ester Sulfonates in Laundry Soap, Detergent Bars, and Powder Detergents for the Asian Hand-Washing Laundry Market, by Rodrigo Olmedo, Detertec S.A., Ecuador; and Synthetic and Aggregate Behavior of Amphiphilic Nickel (II): Complex Coordinating Lipophilic Glyceryldiamine Ligand, by Shuichi Osanai and Takeyuki Makino, Keio University, Japan.

• *Physical, chemical, and environmental aspects.* Additional technical lectures will include Polymorphism, Polytypsin and Martensitic Transition of Petroselinic Acid, by Fumitoshi Kaneko, Osaka University, Japan; A Novel Approach Toward Pollution Abatement in the Palm Oil Industry, by Ah-Ngan Ma, Palm Oil Research Institute of Malaysia, Malaysia; and Wastewater Treatment Method for Oilseed Processing Plants, by K.K. Khoo, De Smet (M) Sdn. Bhd., Malaysia.

• *Raw materials and processing.* The plenary lecture on World Oils and Fats Scenario in the Twenty-First Century will be by B. Bek-Nielsen, United Plantations Bhd., Malaysia. The keynote lecture will be on exotic fats and will be presented by Sushil Goenka, Foods Fats & Fertilisers Ltd., India. Technical papers to be presented will include Soybean Lecithin Production in China, by Gengwang Zhang, Chinese Cereal and Oils Association, China; Improved Agitation Technique in Oils and Fats Modifications, by Anthony Athanassiadis, consultant, Belgium; Optimization of Chemical Transesterification of Palm Oil Using RSM, by T. Haryati, Y.B. Che Man, A. Asbi, I.M. Ghazzali, and L. Buana, Universiti Pertanian Malaysia, Malaysia; Microbial Enzyme Technology in Processing Oilseeds and Oils for Better Food Uses, by D.K. Bhattacharyya, University of Calcutta, India; Production Technology for Tocotrienol- and Tocopherol-Rich Fractions (TRF) from Rice Bran and Industrial Grade Rice Bran, by Z.M. Beg, M. Minhajuddin and J. Iqbal, J.N. Medical College, India; Hydrolysis of Sunflower Oil with Immobilized Lipase, by S.P. Chaurasia, S.R. Patwardhan, and M.K. Trivedi, Indian Institute of Technology, India; Kinetics of *trans* Isomer Fatty Acid Formation During Heating, by K. Kovari, J. Denise, F. Zwoboda, Z.S. Kemeny, K. Recseg, Cereol; and G. Henon, Lesieur Alimentaire, Hungary; Physical Refining of Rapeseed Oil, by J. Cmolik, W. Schwarz, and Z. Svoboda, Setuza a.S., Czech Republic; Hexane, Heptane and Isopropyl Alcohol as Extraction Solvents for Soybean Flakes: Extraction Rate and Oil Quality, by Aytac Saygum Gummuskensen and Fabri Yemiscioglu, Ege University, Turkey; Electro-Filtration—A New Technology for Vegetable Oil Purification After Bleaching, by Gunter Borner, ÖHMI Forschung und Ingenieutechnik GmbH, Germany; Softcolumn Technology—Experience from the Field, by Olle Sternberg, Alfa Laval Separation AB, Sweden; Fat Crystallization with Stirring Surface—Theory and Practice, by Klaus Weber and Thomas Wilner, Krupp Extractionstechnik GmbH, Germany; New Innovation and Improvements in the Edible Oil Industry, by Z. Leibovitz and Z. Balicer, HLD Ltd., Industrial Engineering Co., Israel; The Influence of Crude Palm Oil Quality on Bleachability and Final Color After Deacidification, by Werner Zschau, Süd-Chemie AG, Germany; and A Review of Borohydride Purification of Triglyceride and Natural Product-Based Surfactants, by Michael M. Cook, Morton International Inc., United States.

The schedule for poster presentations will be published in the program distributed at the meeting.

A registration/information brochure containing the complete tentative technical program is available from the ISF Secretariat, 1608 W. Broadmoor Dr., Champaign, IL 61821 (phone: 217-359-2344; fax: 217-351-8091; e-mail: meetings@aocs.org).

serve as synthons in the synthesis of chiral pharmaceuticals and agrochemicals (9).

Triacylglycerols (TAG), natural lipase substrates, are insoluble in water and naturally self-associate to form monomolecular films, micelles or emulsions. Because of this self-association, lipase lipolysis takes place at the lipid/water interface. Because lipolysis is an interfacial reaction, typical enzyme reaction kinetics cannot be applied. Among the publications dealing with this subject, Sarda and Desnuelle (10) showed that the fundamental difference between esterases and lipases is the interfacial activation displayed by lipases which corresponds to their greater activity on aggregated substrates. It was suggested that this phenomenon arises from a change in the conformation of the lipase at the oil/water interface. Within the past few years, molecular modeling and X-ray crystallography have led to the elucidation of the three-dimensional structure of several lipases (11–14). A common feature of the lipases studied so far is that their active site is typically buried under a hydrophobic lid. During lipolysis, interfacial activation results from a change in the lid position which opens by penetrating into the lipid phase, making the active site accessible to the substrate. This interfacial activation is a characteristic of lipases and, as stated previously, differentiates esterases and lipases. However, enzymes with lipolytic activity have been identified that do not display this phenomenon, for example *Fusarium solani* cutinase (15), and the lipases of *Pseudomonas aeruginosa* (9) and *Candida antarctica* B lipase (16). Their lack of interfacial activation is attributed to the absence of a lid in the conformation of these enzymes, and hence they constitute a bridge between lipases and esterases. Therefore, it can be concluded that an absence of interfacial activation is not sufficient to distinguish lipases from esterases. Accordingly, it is preferable to define lipases as esterases that are able to catalyze the hydrolysis of long-chain TAG.

Enantiomers: molecules that are nonsuperimposable mirror image isomers.
Chiral: the property of nonsuperimposability of an object on its mirror image is called *chirality.*
Racemic mixture: a mixture of equal amounts of enantiomers.
Diastereoisomers: compounds that are stereoisomers but not enantiomers.
Prochiral: this term is applied to a molecule with two similar atoms or groups that results in a chiral compound when either is replaced by a third different group or atom. Glycerol is one such prochiral compound.

Lipase specificities

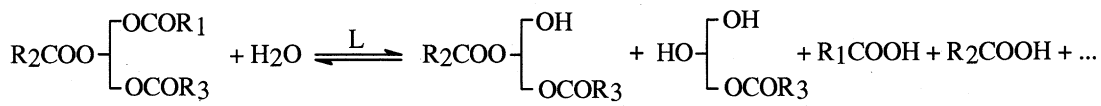
Lipases can be divided generally into five different groups (Table 1): (a) substrate selective lipases; (b) positionally selective (or regioselective) lipases; (c) nonselective lipases; (d) fatty acid selective (or acylselective) lipases; and (e) stereoselective (or *sn*-glycerol specific) lipases.

Substrate specific lipases. Acylglycerols are the normal substrates of lipases. This means that lipases are able to catalyze not only the hydrolysis of ester bonds of TAG but also diacylglycerols (DAG), monoacylglycerols (MAG) and even phospholipids (PL). Thus, substrate specificity is defined as the ability of a lipase to hydrolyze preferentially a particular glycerol ester. For example, during digestion, the hydrolysis of TAG is incomplete. The resulting DAG are transformed into MAG, but the hydrolysis of the latter is very slow (17). Accordingly, TAG are the favored substrates of most animal

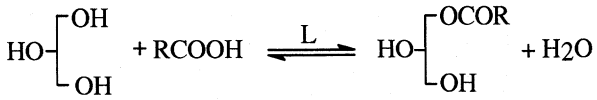
Table 1
Classification and selected examples of lipase specificities

Specificity	Lipase	Reference
Substrate specific		
Monoacylglycerols	Rat adipose tissue	19
Mono- and diacylglycerols	*Penicillium camembertii*	18
Triacylglycerols	*Penicillium* sp.	20
Regiospecific		
1,3-Regioselective	*Aspergillus niger*	22
	Rhizopus arrhizus	21
	Mucor miehei	59
sn-2-Regioselective	*Candida antarctica* A	23
Nonspecific		
	Penicillium expansum	24
	Aspergillus sp.	25
	Pseudomonas cepacia	35
Fatty acylspecific		
Short-chain fatty acid (FA)	*Penicillium roqueforti*	27
	Premature infant gastric	28
cis-9 Unsaturated FA	*Geotrichum candidum*	26
Long-chain unsaturated FA	*Botrytis cinerea*	30
Stereospecific		
sn-1 Stereospecific	*Humicola lanuginosa*	23
	Pseudomonas aeruginosa	23
sn-3 Stereospecific	*Fusarium solani* cutinase	23
	Rabbit gastric	58

Hydrolysis



Esterification



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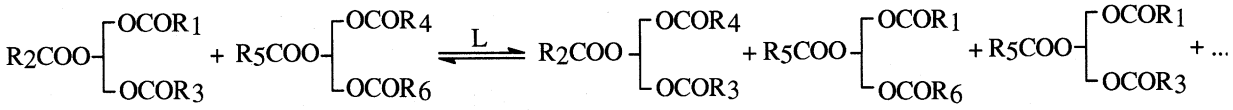
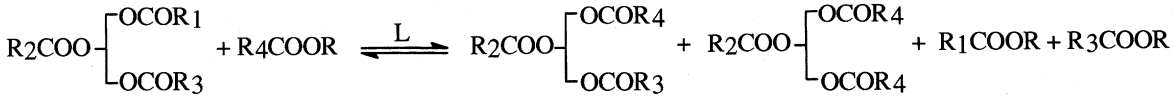
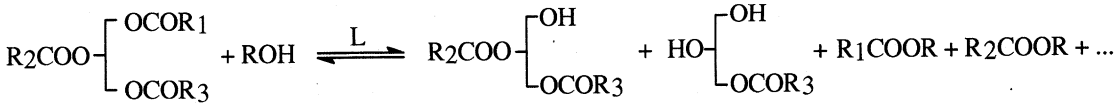


Transesterification



Alcoholysis



Acidolysis

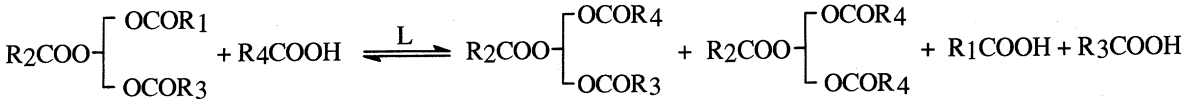


Figure 1. Principal lipase-catalyzed reactions in oil and fat modification

lipases, whereas MAG are poor ones. TAG also are preferred substrates for the large majority of plant and microbial lipases. However, a few lipases are reported to hydrolyze partial glycerides faster than TAG. For example, *Penicillium camembertii* lipase (18) has been described as preferring MAG and DAG with minimal reactivity with TAG. A MAG-specific lipase from rat adipose tissue has been reported that exhibits little activity with DAG and TAG (19). Recently, a lipase from *Penicillium* sp. that discriminates against DAG has been described (20).

Positional specific or regioselective lipases. Regioselectivity is defined as the ability of lipases to distinguish between the two external positions (primary ester bonds) and the internal position (secondary ester bond) of the TAG backbone. During lipolysis of TAG substrates, 1,3-regioselective lipases preferentially hydrolyze the *sn*-1 and *sn*-3 positions over the *sn*-2 position (Figure 2). In this manner, an equimolar mixture of 1,2-DAG and 2,3-DAG are obtained, the subsequent hydrolysis of which leads to 2-MAG. Pig pancreatic lipase, for example, is regarded as being 1,3- regioselective.

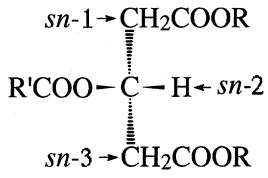


Figure 2. Representation of *sn*-nomenclature for triacylglycerols

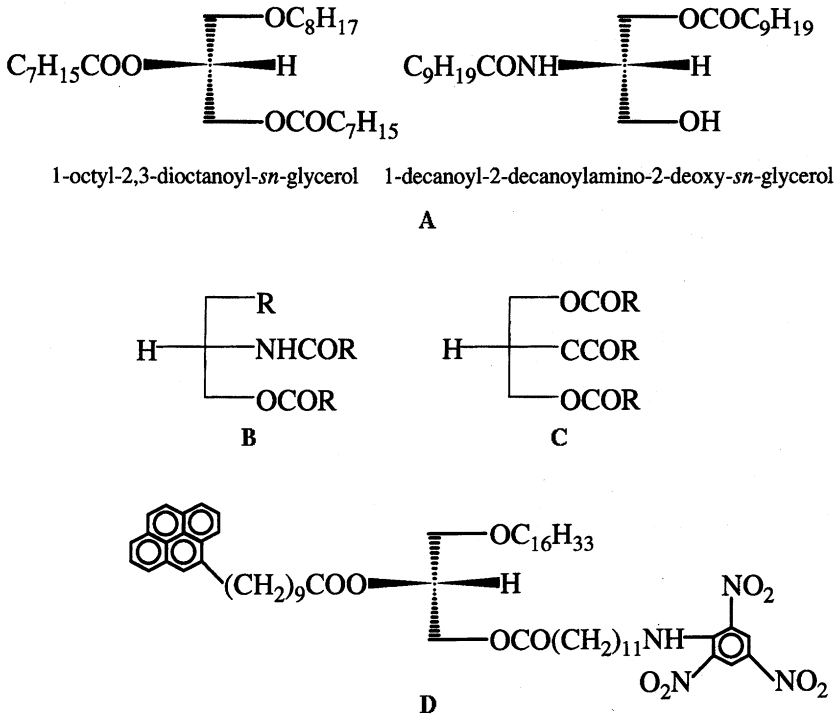


Figure 3. (A) Chiral alkyl acylglycerol substrates used by Ransac *et al.* (51), the enantiomers 3-octyl-1,2-dioctanoyl-*sn*-glycerol and 3-decanoyl-2-decanoylamino-2-deoxy-*sn*-glycerol also were used). (B) General structure for the triacylglycerol analogues used by Mannese *et al.* (52). (C) General structure of the carba analogues used by Berger *et al.* (54), substrates with carbon-carbon bonds at the 1,3-positions and a carbon-oxygen bond at the 2-position also were used. (D) Fluorogenic 1-alkyl-2,3-diacylglycerols used by Zandonella *et al.* (53), the (S) enantiomer 1-trinitrophenylaminolauroyl-2-pyrenedecanoyl-1-*O*-hexadecyl-*sn*-glycerol also was used.

This type of specificity is reported for several microbial lipases, including those produced by *Aspergillus niger* (21) and *Rhizopus arrhizus* (22). True *sn*-2 regioselectivity is very rare and, to the best of our knowledge, only one lipase, namely *C. antarctica* A (23), has been reported that preferentially hydrolyzes the *sn*-2 position of the TAG backbone.

Nonspecific lipases. A number of lipases display little if any positional specificity and hydrolyze all ester bonds in TAG without regard to the particular fatty acyl components of the substrate being hydrolyzed. Examples of nonspecific lipase are numerous and include the lipases from *Penicillium expansum* (24) or *Aspergillus* sp. (25).

Fatty acid specific or acylselective lipases. Lipases also can be specific for a particular fatty acid or, more generally, for a class of fatty acids. Such lipases then will hydrolyze glyceride esters of these acids regardless of their position on the glycerol backbone. The most extensively studied lipase of this type is that from *Geotrichum candidum* which is highly specific for *cis*-9-unsaturated fatty acids (26). Other lipases from different origins, such as *Penicillium roqueforti* (27) and gastric lipases of various animals (28,29) are specific for short-chain fatty acids and show little activity toward long-chain acids. There are lipases that also are specific for other fatty acids: for example, *Botrytis cinerea* lipase is specific for long-chain unsaturated fatty acids (oleic and linoleic acids) (30).

Stereospecific lipases. This type of specificity is defined as the ability of lipases to distinguish between the *sn*-1 and *sn*-3 positions of TAG. Reports for this type of specificity are relatively recent, with Jensen *et al.* (31) reporting an *sn*-3 stereospecificity for human lingual lipases. More recently, Rogalska *et al.* (23) determined the stereospecificity of a large number of animal and microbial lipases. These latter authors reported *sn*-1 stereospecificity for lipases from *Humicola lanuginosa, Pseudomonas fluorescens,* and lipoprotein lipase from bovine milk and *sn*-3 stereospecificity for *C. antarctica* B, dog gastric lipase, and *F. solani* cutinase.

Owing to the above specificities, lipases are enzymes with unique catalytic properties, and their use is becoming increasingly important in various industrial processes (e.g., formulation of simulated dairy products, modification of oils and fats). In addition, their application in the synthesis of chiral compounds is becoming popular. Because of this, several methods for determining lipase specificities have been developed.

Determination of lipase specificities

Methods to determine lipase specificities are numerous and varied, depending upon the type of specificity one wishes to examine (Table 2). Moreover, it is necessary to understand that lipase specificities should be studied to a limited extent of hydrolysis (typically <20% hydrolysis) to avoid complications arising from acyl migration or enzymatic hydrolysis of the partial glyceride products which may lead to erroneous interpretations of the data.

Hydrolysis of natural substrates. The easiest methods use natural substrates, such as oils and fats, in determining positional and fatty acid specificities. The hydrolysis products, once isolated, are analyzed by a number of analytical techniques. Quantitation and comparison of the 1,2-DAG and 2,3-DAG products with the 1,3-DAG products give information on the lipase preference toward primary ester bonds at the 1- and 3-positions or the secondary ester bond at the 2-position of the glycerol backbone. For example, Riaublanc *et al.* (32) used rapeseed oil as a substrate for determining the specificity of *C. parapsilosis*. The hydrolysis products were analyzed by thin-layer chromatography (TLC). The authors found a majority of 1,3-DAG and concluded a

Table 2
Selected examples of methods for determining lipase specificities

Method	Lipase studied	Reference
Hydrolysis of natural substrates		
Vegetable oils	*Aspergillus* sp.	25
Milk fats	Seal gastric	34
High erucic rapeseed oil	*Pseudomonas cepacia*	35
	Geotrichum candidum	
Hydrolysis of homogeneous TAG		
Determination of acyl selectivity	*Bacillus* strain A30-1	37
	Vernonia galamensis	39
	Corn, castor bean, rapeseed, elm seed	40
	Oat seed	42
Regioselectivity	Porcine pancreatic, *Rhizopus delemar*, *R. niveus*, etc.	43
Use of homogeneous TAG as prochiral substrates to determine stereoselectivity	Animal and microbial	23,44
Use of fatty acid esters		
Hydrolysis of methyl esters	*Mucor miehei*	45
Transesterification with ethyl esters	*Candida antarctica*	46
Hydrolysis of umbelliferone esters	*Botrytis cinerea*	30
Hydrolysis of *p*-nitrophenyl esters	Sunflower seed	47
Esterification of FA with alcohols	Microbial	48
Hydrolysis of synthetic TAG analogues		
Radio-labeled mixed alkylacylglycerols	Rat lingual, rat adipose tissue	50
Alkylacylglycerols	Animal gastric and pancreatic	51
Alkylacylglycerols acylglycerols	*Fusarium solani*	57
Fluorogenic alkylacylglycerols	Microbial	53
Triglyceride carba analogues	Animal and microbial	54
Hydrolysis of heterogeneous synthetic TAG		
Chiral diacid triglycerides	Premature infant lingual	31
Racemic triglycerides	Premature infant gastric	28
Racemic triacid triglycerides	Microbial	55
Chiral and racemic triacid triglycerides	Animal, microbial, or plant	58–60

faster hydrolysis rate for the fatty acids at the *sn*-2 position. It is worth noting, however, that other studies considered possible acyl migration and did not corroborate this type specificity for this lipase (33). Iverson *et al.* (34) studied seal gastric lipase specificity on milk fat substrates from the same species. They first determined the fatty acid distribution and composition of the fat and showed that this fat is rich in polyunsaturated fatty acids (PUFA). In addition, the PUFA were located mainly on the 1- and 3-positions of the TAG backbone. Given the fact that hydrolysis of this substrate by seal gastric lipase preferentially released PUFA, the authors concluded a 1,3-regioselectivity for the enzyme. Fu *et al.* (25) used different natural substrates (olive oil, soybean oil, mink fat, coconut oil) to study an *Aspergillus* sp. lipase. The partial glycerides were separated by TLC, methyl esters were prepared, and the fatty acid distribution determined by gas chromatography (GC). The results showed that this lipase was nonspecific.

Natural substrates also can be employed in determining whether a lipase has a particular fatty acid specificity. Sonnet *et al.* (35) studied the hydrolysis of high erucic acid rapeseed oil by *Pseudomonas cepacia* and *G. candidum*. It was found that in hydrolysis reactions, *G. candidum* clearly discriminated against erucic acid and other fatty acids with chain lengths greater than C18 whereas *Pseudomonas cepacia* had no chain-length preference. Lipase specificities obtained from methods involving natural substrates, however, can be ambiguous because it is difficult to

determine whether the results are due to the specificity of the enzyme used or are the consequence of a particular fatty acid distribution and composition of the natural TAG substrate. Ruminant lipases are one example because when their specificities were evaluated on milk fat, in which a large proportion of its short-chain fatty acids are located in the *sn*-3 position (36), there was no differentiation made between acyl and *sn*-selectivity of the lipase.

Hydrolysis of homogeneous TAG. Homogeneous TAG (monoacid TAG) are used in determining acylselectivities of lipases by comparing hydrolysis reactions on substrates that vary in the types of the fatty acids (chain length, unsaturation, etc.). *Bacillus* strain A30-1 lipase was studied (37) on a series of homogeneous TAG. It was observed that tributyrin hydrolysis was fastest and that the enzyme had little or no activity toward long-chain TAG. Bertolini *et al.* (38) studied two recombinant lipases from *G. candidum* and evaluated their specificities using several homogeneous substrates that varied both in chain length and in the number and position of double bonds. One lipase showed higher activity toward long-chain unsaturated fatty acids whereas the second lipase had a preference for short-chain saturated fatty acids. A number of plant lipases have been studied by this method, the lipase from *Vernonia galamensis* being one example (39). The major fatty acid of this seed oil is vernolic acid. By using trivernolin as substrate and comparing it with other homogeneous TAG, it was found that this plant lipase had a selectivity for vernolic acid. The specificities of other plant lipases studied with homogeneous TAG include: corn lipase on trilinolein and triolein, castor bean lipase on triricinolein, rapeseed lipase on trierucin, and elm seed lipase on tricaprin (40). Finally, Giordani *et al.* (41) worked with a latice lipase from *Carica papaya* and showed its specificity for tributyrin. Piazza *et al.* (42) studied the fatty acid selectivity of oat seed lipase using long-chain unsaturated homogeneous TAG. They showed that the selectivity exhibited by this lipase is similar to that of the *G. candidum* except that the oat seed lipase attacks elaidate, a fatty acid group with a *trans* double bond whereas the *G. candidum* lipase strongly discriminates against *trans* fatty acids.

This method of demonstrating acyl selectivities of lipases also has shortcomings. Lipase specificities in lipolysis reactions are due not only to the biocatalyst but also to the substrate and more particularly to the interfacial quality. Faster hydrolysis of tributyrin compared to triolein or tripalmitin does not necessarily imply that the lipase is butyric acid-selective; it also can be a consequence of differences between emulsions, micelles or films formed with individual TAG. Data from experiments using detergents or other surfactants also need to be very carefully interpreted in this regard. Moreover, homogeneous TAG have differing melting points depending on their acyl chain composition: Long-chain saturated TAG are solids at room temperature whereas unsaturated, short- or medium-chain triglycerides are liquid. In general, solid TAG are hydrolyzed by lipases more slowly than liquid TAG. Accordingly, with this method it is important to conduct hydrolysis reactions under conditions where all substrates are in the same physical state. The weakness of this method can be overcome with the use of simple alkyl fatty acid esters which are liquid under the usual experimental conditions (30–60°C).

Homogeneous TAG also are used to evaluate positional specificities by a comparison of 1,2-(2,3)-DAG and 1,3-DAG obtained by partial hydrolysis. Ota *et al.* (43) hydrolyzed triolein with different lipases and used chiral-phase high-performance liquid chromatography (HPLC) to separate and quantify the different reaction products after conversion to the diastereoisomeric dinitrophenylurethanes. Probably the most original and relevant work with homogeneous TAG is their use as substrates for the determination of a lipase's *sn*-stereospecificity. The primary positions of a homogeneous TAG are stereo nonequivalent because they are prochiral compounds in that exchange of either primary acyl group with a second acyl group results in an *sn* TAG. Thus, lipases are able to distinguish between the *sn*-1 and *sn*-3 positions of these substrates (Figure 2). The first determination of a lipase's *sn*-stereospecificity using homogeneous substrates was based on the measurement of the specific optical rotation of the isolated enantiomeric DAG (31). Recently, Rogalska *et al.* (44) proposed a more efficient method in which the enantiomeric DAG obtained from the lipolysis of trioctanoin or triolein were converted into diastereoisomeric carbamates, by derivatization with R (+) phenylethylisocyanate and the diastereomers were separated by HPLC. The *sn*-specificity of several animal or microbial lipases was determined by this method (23).

Use of fatty acid esters. As previously noted, a disadvantage of using homogeneous substrates in determining fatty acid specificities of lipases is the different physical states of TAG. The use of simple alkyl esters may eliminate this difficulty as fatty acid esters are liquid under typical experimental conditions. To determine acyl selectivities two strategies can be employed with this type of substrates: hydrolysis of alkyl fatty esters or esterification of free fatty acids. Aggelis *et al.* (45) used methyl esters of borage oil as substrates using *Mucor miehei* lipase and concluded that this lipase was specific for short-chain fatty acids and for acids with minimal unsaturation. Huang and Akoh (46) measured the incorporation of n-3 PUFA ethyl esters into different oils using a *C. antarctica* lipase and observed that this lipase favored n-3 PUFA. Other types of esters can be used in determining acyl specificity of lipases. For example, the specificity of an extracellular lipase from *B. cinerea* was studied using umbelliferous esters of fatty acids (30). *p*-Nitrophenyl esters of varying chain length were used to measure the specificity of several enzymes (47). Jachmanian *et al.* (48) studied the substrate selectivity of several lipases with fatty acids in an esterification reaction with *n*-butanol. The substrates were fatty acids having *cis*-4, *cis*-6, and *cis*-8 unsaturation; fatty acids having other functional groups such as cyclopentenyl, hydroxy and epoxy, were stud-

ied also. The lipases examined included microbial lipases from *C. cylindracea*, *R. arrhizus* and *M. miehei*, pig pancreatic lipase, and a lipase from rape. They found that all these lipases strongly discriminated against *cis*-4, *cis*-6, or *cis*-8 unsaturated fatty acids whereas esters of fatty acids with other functional groups were well accepted as substrates. Similarly, Mukherjee *et al.* studied several commercially available lipases with regard to their substrate specificity in the esterification of fatty acids having *cis* double bonds at specific positions of the acyl chain (49). They showed that a common feature of the studied lipases was that fatty acids having the first double bond from the carboxylic end as a *cis*-4, *cis*-6 or a *cis*-8 unsaturation were strongly discriminated against compared to common fatty acids.

Hydrolysis of synthetic TAG analogues. One difficulty in defining lipase selectivity with methods employing natural or homogeneous TAG substrates is acyl migration, which can lead to erroneous interpretations. A major advantage of the use of TAG analogues, e.g., mixed alkylacyl glycerols, is that it removes this complication since acyl migrations are not encountered with these substrates. Paltauf *et al.* (50) were among the first to use mixed alkylacylglycerol substrates. Ransac *et al.* (51) synthesized a series of mixed alkylacylglycerols (Figure 3A) and evaluated the specificity of several gastric and pancreatic lipases. Recently other methods involving glyceride analogues have been developed. Mannese *et al.* (52) studied the behavior of cutinase from *F. solani pisi* in hydrolyzing TAG analogues where one primary acyl function was replaced by an alkyl group and the *sn*-2 acyl function by an amide group (Figure 3B). Zandonella *et al.* (53) synthesized enantiomeric 1-*O*-alkyl 2,3-diacyl-*sn*-glycerol and 3-*O*-alkyl 1,2-diacyl-*sn*-glycerols that contained pyrene as a fluorescent probe and a trinitrophenylamino residue as a fluorescent quencher (Figure 3D). The initial substrates have low fluorescence, but during enzymatic lipolysis the release of fatty acyl chains leads to increased fluorescence from which the rate of lipolysis can be measured. The two enantiomeric analogues were used to evaluate the activity and stereopreference of several lipases. Finally, Berger *et al.* (54) synthesized carba analogues of TAG, e.g., in Figure 3C the secondary oxygen atom of the glycerol moiety has been replaced by a carbon atom. The regioselectivity of a number of lipases was evaluated by comparing the hydrolysis rate of two TAG carbon analogues in which the substitution of the C-O ester bond by a C-C bond was at the 1- and 3-positions for the first TAG analogue, and at the 2-position for the second TAG analogue.

It is quite possible that the specificity of a lipase on a TAG analogue may not be the same as for natural TAG substrates.

As noted, the use of glyceride analogues is very useful since it avoids the complication of acyl migration. However, these substrates are not true TAG and it is quite possible that the specificity of a lipase on a TAG analogue may not be the same as for natural TAG substrates.

Hydrolysis of heterogeneous synthetic TAG. A few methods use synthetic chiral (*sn*) TAG as substrates for determining lipase specificities. Although their synthesis is difficult, they are very useful in evaluating lipase specificity since they are truly defined TAG and offer a major advantage over methods involving natural substrates because the method removes the ambiguities associated with fatty acyl distributions and compositions of the natural substrates. Jensen *et al.* (31) used the chiral TAG, 1-oleoyl-2,3-palmitoyl-*sn*-glycerol, and 1-palmitoyl-2,3-oleoyl-*sn*-glycerol as substrates to study premature infant lingual lipase. They observed that the *sn*-3 ester bond was hydrolyzed about four times faster than the *sn*-1 ester bond. Recently, these same authors (28) studied premature infant gastric lipase using racemic acyl TAG, that varied in chain length as substrates. The authors concluded there was a short-chain specificity of the studied lipase and also noted a selectivity for the primary ester bonds. Sonnet and Gazzillo (55) used the racemic TAG, namely 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl-*rac*-glycerol to evaluate the positional specificity of several commercially available lipases. After the hydrolysis reaction, partial glycerides and free fatty acids were recovered and analyzed as methyl esters by GLC. *Candida cylindracea* lipase showed a slight preference for hydrolyzing palmitic and stearic residues at the 1- and 3-positions. The behavior of *G. candidum* lipase was comparable to *C. cylindracea*. The former lipase, however, was highly selective for the oleoyl residue at the 2-position of this TAG. *Rhizopus delemar* lipase appeared to be the most 1,3- regioselective since only 3–4% oleic acid was released at 51% hydrolysis. Recently, a new method was developed involving chiral and racemic synthetic TAG. The chemical route to these compounds (56) gives substrates with fatty acid distributions and compositions that are highly defined and checked by a method favoring the analysis of short-chain fatty acids (57). The hydrolysis of the chiral TAG, 1-butyroyl-2-stearoyl-3-palmitoyl-*sn*-glycerol, and of its racemic equivalent, 1(3)-butyroyl-2-stearoyl-3(1)-palmitoyl-*rac*-glycerol, allowed for an accurate determination of a lipase's specificity. The method was applied to the study of several lipases from different origins (58). For example, an *sn*-1 stereospecificity was determined for *P. aeruginosa* and an *sn*-3 specificity for rabbit gastric lipase. The same method was extended to the study of a plant lipase from *Carica papaya* which was determined as an *sn*-3 stereospecific lipase (59). Pregastric lipases from young ruminants also were studied by this method (60). It was concluded that kid, calf, and lamb pregastric

lipases have a combination of short-chain specificity and *sn*-3 stereospecificity. Kid pregastric lipase had the highest acyl selectivity and *sn*-3 stereopreference and calf lipase the poorest acyl selectivity.

Applications of lipase specificities

The use of lipases is becoming increasingly important in a number of applications such as in the pharmaceutical, dairy, and cosmetic industries. This discussion will focus on the use of lipase specificities in the classical reactions of oils and fats bioconversion (Figure 1). Lipases have distinct advantages compared to classical chemical catalysts: (a) enzymes function under mild reaction conditions, thus avoiding the formation of side products, (b) enzyme-catalyzed reactions are more efficient and easier to control, and (c) the unique specificities of lipases allow the design of synthesis routes that predetermine product structure and distribution, whereas chemical catalysts generally lead to random reaction product mixtures. Thus, the use of lipases makes it feasible to modify the fatty acid composition and TAG distribution of an oil or fat so as to obtain new products with predetermined physical and chemical properties.

Use of nonspecific lipases. As noted, the use of nonspecific lipases in oil and fat bioconversions has advantages over chemical catalysts. Although both reactions are random in nature, lipases offer better kinetic control and milder reaction conditions. These can be important, because steam splitting that occurs at high temperatures can lead to discoloration and degradation of the liberated fatty acids, especially PUFA (2).

Use of fatty acid-specific lipases. Fatty acid-specific lipases may be used in any of the three classical reactions of oil and fat bioconversion, namely, hydrolysis, transesterification, and interesterification. The reaction can be based on a lipase's preference for a particular fatty acid or on a lipase's discrimination against a particular type of fatty acid to obtain products enriched in one or a particular type of fatty acid. The specificity of *G. candidum* lipase is such that it discriminates against fatty acids with chain lengths greater than 18 carbons and/or fatty acids with unsaturation at the n-6 and n-12 positions. This specificity has been applied in the enrichment in different oil fractions of a given fatty acid, especially PUFA. For example, the free fatty acid fraction of borage oil has been concentrated in γ-linolenic acid in esterification reactions catalyzed by this lipase (61). Futhermore, a two-step enzymatic procedure for the isolation of erucic acid from rapeseed oil based on chain-length discrimination of this lipase has been developed (62). Similarly, the ability of *M. miehei* and *C. cylindracea* lipases to discriminate against the n-3 family of PUFA (e.g., eicosapentaenoic acid) has been exploited for the selective harvesting of PUFA from fish oils (63).

Short-chain preference is also a lipase specificity that can be exploited on an industrial scale. Such lipases may be used in the production of low-calorie structured TAG (64) or in the dairy industry to obtain specific flavor components by the release of short- or medium-chain fatty acids from milk fat. The short-chain specificity of a lipase from *C. antarctica* may prove useful in interesterification reactions to increase the ratio of medium-chain TAG (MCT) in different oils (65). The latter TAG are of interest due to their unique nutritional properties.

Plant lipases are the subject of recent interest because of their particular fatty acid specificities as well as their apparent relative ease of isolation and lower cost. For example, rapeseed lipase, which discriminates against fatty acids with a *cis*-6 or *cis*-4 double bond, has been used for the enrichment of these fatty acids (66). Recently, a patent has appeared describing the use of a latex lipase from *Carica papaya* to modify the rheological and/or nutritional properties of milk fats (67). The strong acyl selectivity of this enzyme has been used in inter- and transesterification reactions of acylglycerols to selectively remove the short-chain fatty acids and to replace them by, for example, long-chain saturated fatty acids. This lipase is particularly useful since, in addition to its strong short-chain acyl selectivity, it exhibits an *sn*-3 stereospecificity.

Use of 1,3-regioselective lipases. 1,3-Regioselective lipases have significant advantages compared to chemical catalysts. With the latter, interesterification reactions are typically carried out with refined fats and oils, since unwanted side reactions may occur with unrefined substrates. On the other hand, lipase-catalyzed interesterifications can be applied to partially refined substrates. Chemical catalysts also give a random distribution of fatty acids in the restructured oil or fat, whereas lipases limit this random distribution. Moreover, use of a 1,3-regioselective lipase does not alter the fatty acid composition at the *sn*-2 position of TAG, which are typically rich in essential PUFA. Because 2-MAG are the main carriers of PUFA through the internal tissues, it is useful to maintain these essential fatty acids in the *sn*-2 position.

Muderwha *et al.* (68) did a comprehensive study of 1,3-regioselective lipase-catalyzed interesterification reactions and clarified some important points relative to this reaction. The transesterification reaction was studied with different lipases, produced by *C. deformans*, *R. arrhizus*, and *M. miehei* and compared to the hydrolysis reactions catalyzed by the same lipases. There appeared to be no relationship between lipolytic activity and interesterification activity (68) in that a lipase with a high lipolytic activity may or may not have interesterification activity. The effect of water activity on interesterification also was evaluated (69). For lipase interesterification reactions, a given water activity is needed to obtain good lipase activity and minimize competitive hydrolysis reactions. The final part of this study involved determining the preservation with time of the 1,3-regioselectivity of the biocatalyst during an interesterification reaction (70) between copra oil and methyl stearate and measuring the incorporation of stearic acid in the *sn*-2 position. It was found that for the lipases studied, their 1,3-selectivity was retained for at least 12 hours, long enough to ensure that no acyl migration occurred during the interesterifi-

cation reaction. Finally, this work was applied to the interesterification of palm oil with soy oil in an effort to reduce the solid fat content of palm oil (71).

1,3-Regioselective lipases have been used widely on an industrial scale to obtain new fats with nutritionally improved properties. One research group used such a lipase for the development of a novel TAG with a fatty acid distribution similar to human milk fat by interesterifying tripalmitin with PUFA (7). The result was a TAG rich in palmitic acid in position *sn*-2 and with PUFA on the 1,3-positions. This technology was extended by the same research group to produce milk-fat substitutes for infant formula obtained solely from vegetable sources. The product with a saturated fatty acid in position *sn*-2 was shown to improve digestibility and enhance the absorption of other nutrients. Similarly, TAG with PUFA at the *sn*-2 position and medium-chain fatty acids at the 1,3-positions can be produced by enzymatic interesterification. Such MCT have unique properties in that they are more rapidly absorbed than usual TAG (64). A recent paper describes the high yield synthesis of 1(3)-MAG and 1,3-DAG by using highly 1,3-regioselective lipase-catalyzed esterification of glycerol and vinyl esters of fatty acids (72).

Other specificities. To our knowledge, no large-scale applications involving lipase with specificities other than 1,3-regioselectivity or acyl selectivity have been developed. It is evident that the discovery of a truly *sn*-2 regioselective lipase would be of great interest in oil and fat bioconversions since this position is typically very rich in essential fatty acids in natural oils. Stereospecific lipases also are of interest for modifying either the *sn*-1 or *sn*-3 position of TAG. These lipases would be useful for preparing asymmetric substrates, such as milk fats (where short-chain fatty acids are located at the *sn*-3 position). One can envision, for example, the use of an *sn*-3 stereospecific lipase for the transesterification of vegetable oils with short-chain fatty acids to give products that mimic the fatty acid distribution and composition of natural milk fats.

Conclusion and perspectives

The use of lipases in oils and fats bioconversion has many advantages over classical chemical catalysts. Lipases operate under milder reaction conditions over a range of temperatures and pressures that minimize the formation of side products. One of their most attractive features is the unique specificities that they often display. Because several methods are available for analyzing lipase reactions, it is now possible to classify lipases based on their specificities and to select a particular enzyme for a desired application. The present challenges are to understand the mechanisms of lipase-catalyzed reactions and to answer the question: What are the bases for a lipase specificity? The recent advance in molecular modeling of protein three-dimensional structure is an important step in answering this question. Within a few years, protein engineering and mutagenesis techniques will most likely lead to the availability of "tailor-made" lipases with improved properties (activity, pH range, thermostability, etc.) and designed specificities. Other problems, such as enzyme cost and reusability, are still a challenge but oil and fat bioconversions will undoubtedly become important processes in the future.

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